# MICROBIOLOGICAL ANALYSIS OF PERIODONTAL POCKET (SUBGINGIVAL PLAQUE) AND CORONARY ATHEROMATOUS PLAQUE IN ATHEROSCLEROTIC PATIENTS

*A Dissertation Submitted*

*in partial fulfillment of the requirements*

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**PERIODONTOLOGY**


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# CERTIFICATE

This is to certify that **Dr. V.DHIVYAPRIYA,** Post Graduate student (2007 – 2010) in the Department of Periodontology, Tamil Nadu Government Dental College and Hospital, Chennai – 600 003, has done this dissertation titled **'MICROBIOLOGICAL ANALYSIS OF PERIODONTAL POCKET (SUBGINGIVAL PLAQUE) AND CORONARY ATHEROMATOUS PLAQUE IN ATHEROSCLEROTIC PATIENTS'** under our direct guidance and supervision in partial fulfillment of the regulations laid down by the **Tamil Nadu Dr. M.G.R. Medical University,** Chennai – 600 032 for **M.D.S.,** (Branch II) **Periodontology** degree examination.

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# LIST OF PHOTOGRAPHS

## LIST OF FIGURES



## LIST OF TABLES

# CONTENTS

# LIST OF ABBREVIATION

| | |
|---|---|
| **BMI** | Body Mass Index |
| **CAD** | Coronary Artery Disease |
| **CAL** | Clinical Attachment Level |
| **CHD** | Congestive Heart Disease |
| **CPK** | Creatinine Phosphokinase |
| **CRP** | C-reactive Protein |
| **CVD** | Cardio Vascular Disease |
| **DNA** | Deoxyribonucleic Acid |
| **EDTA** | Ethylene Diamine Tetra Acetic Acid |
| **ELISA** | Enzyme Linked Immunosorbent Assay |
| **HDL** | High Density Lipo Protein |
| **Hsp** | Heat Shock Protein |
| **IFA** | Indirect Immunofluorescence Assay |
| **Ig** | Immuno Globulin |
| **IL** | Inter Leukin |
| **LAD** | Left Ascending Distal Artery |
| **LDH** | Lactate Dehydrogenase |
| **LDL** | Low Density Lipo Protein |
| **LIMA** | Left Internal Mammary Artery |
| **PCR** | Polymerase Chain Reaction |
| **PI** | Plaque Index |
| **PPD** | Probing Pocket Depth |
| **RNA** | Ribonucleic Acid |
| **RPM** | Rotations per minute |
| **TAE** | Tris Acetic EDTA |
| **TBS Buffer** | Tris Buffered Saline Buffer |
| **TE Buffer** | Tris EDTA |
| **UV** | Ultra Violet |

# ABSTRACT

**BACKGROUND:**

It has been suggested that chronic infections may predispose to cardiovascular disease. The relationship between periodontal disease and cardiovascular disease has been a subject of increasing research in recent years. The isolation and identification of periodontal bacteria from atheromatous plaque can contribute to our knowledge of this vascular disease.

**AIM:**

The aim of this study was to isolate and identify periodontal pathogens from the sub gingival plaque of different patients and to compare them with the microorganisms detected in the atheromatous plaque obtained from the same patients.

**METHODS:**

The study was performed on twenty five individuals with atherosclerosis. Atheromatous Plaques was collected from the patients during endarterectomy and the subgingival plaque was collected from the same patients with clinical attachment level $\geq$4mm. Total DNA isolation was done, the presence of 16S rDNA for *P.Gingivalis,T.Denticola, T.Forsythia and A actinomycetem comitans* was determined using PCR technique. The products were sequenced and they were compared with type strains from Gen Bank by blast to confirm their sequence homology. McNemar's Test was used for statistical analysis.

**RESULTS:**

The DNA of atleast one of the target bacteria was detected in 96% of the sub gingival Plaque samples. The prevalence of *P.gingivalis, T. Denticola, and T. Forsythia* was 92%, 80% and 96% respectively. The prevalence of *P.Gingivalis, T.Forsythia, and T.Denticola* in atheromatous plaque samples was 64%, 56% and 76% respectively. The DNA of periodontal bacteria except *A.actinomycetemcomitans* was detected in both sub gingival samples and atheromatous samples. In 16 of 25 cases, *P.gingivalis* was present in both sub gingival samples and atheromatous plaque samples at P value of 0.008. In 19 cases, *T.denticola* was present in both sub gingival samples and atheromatous plaque samples at P value of 0.031 and T.forsythia were present in both subgingival and atheromatous plaque samples in 14 cases at P value of 0.016.

**CONCLUSION:**

The presence of *P.Gingivalis, T. Denticola, T. forsythia* in both atheromatous plaques and the subgingival plaque of an individual could indicate a role for periodontal pathogenic bacteria in atherosclerosis disease process.

# INTRODUCTION

Several hundred different species of bacteria inhabit the oral cavity. Among these periodontal diseases associated bacteria adhere to and colonize the periodontal pocket.

Periodontitis is a bacterially induced, localized, chronic inflammatory disease, destroys connective tissue and bone that support the teeth. Periodontitis begins with a microbial infection, followed by a host mediated destruction of soft tissue caused by hyper activated or primed leukocytes and the generation of cytokines, eicosanoids and matrix metalloproteinases that causes clinically significant connective tissue and bone destruction (**Kornman KS et al 1997)** [51]**.**

Bacterial accumulation on the teeth are essential to the initiation and progression of periodontitis .Cells that mediate immunity, such as neutrophils, play a major role in the host response against invading periodontopathic organisms. When bacterial biofilms on the teeth are not disrupted on a regular basis, ecologic changes lead to the emergence of a small set of gram negative anaerobic bacterial species, including *Porphyromonas gingivalis*, *Treponema denticola and Tannerella forsythia*, which are consistently associated with periodontitis. These bacteria activate many host immunoinflammatory processes and disrupt host mechanism involved in bacterial clearance and are considered pathogens in periodontitis.

Chronic infection can be one of the contributing factors involved in atherosclerosis (**Ross. R 1999**)[75]. It has been suggested that periodontal pathogens can penetrate gingival tissues and enter the blood stream (**Lamont RJ et al 1995**[53]**, Fives – Taylor.P.D. et al 1995**[29]) and may also induce a continuous benign bacteraemia (**Sconyers JR et al 1973**[79]) that can allow species associated with periodontal diseases to migrate to atheromatous plaques.

This study was undertaken to ascertain the presence of DNA of periodontal bacteria, *Tannerella forsythia, Porphyromonos gingivalis, Treponema denticola, Aggregatibacter actinomycetemcomitans,* in coronary atheromatous plaque and to compare them with periodontal bacteria from the subgingival plaque samples obtained from the same patients.

# AIM AND OBJECTIVES

The aim of this study was to isolate and identify periodontal pathogens from the subgingival plaque of different patients and to compare them with the microorganisms detected in the atheromatous plaque.

For the purpose, following objectives were undertaken.

1. **16S rDNA** screening of *Porphyromonas gingivalis, Treponema denticola, Aggregatibacter actinomycetemcomitans, Tannerella forsythia*, in subgingival plaque samples of atherosclerotic patients by polymerase chain reaction

2. **16S rDNA** screening of *Porphyromonas gingivalis, Treponema denticola, Aggregatibacter actinomycetemcomitans, Tannerella forsythia*,in atheromatous plaque samples of the same patients by polymerase chain reaction

3. To find out the statistical significance of organisms detected in subgingival plaque and atheromatous plaque

# REVIEW OF LITERATURE

## PERIODONTITIS AND ITS BACTERIAL ETIOLOGY

**Breasted J H in 1930[12]** stated, periodontal disease was the most common of all the diseases found in the embalmed bodies of ancient Egyptians. **Armitage GC in 1999[4]** reported that, numerous forms of periodontal disease are found in adult populations, characterized by different rates of progression and different responses to treatment.

According to analysis of **NHANES III data[47]** for adults 30yrs and older, the prevalence of periodontal attachment loss, ranging from a high of 99% for a threshold greater than 1mm to a low of 7% for a threshold greater than 7mm. At a threshold of greater than 3mm, the prevalence of attachment loss in at least 1 site in the mouth was 53%. The prevalence of attachment loss increased steadily with age from a low of 35.5% for the 30-39 year old groups to a high of 39.2% for the 80-90 year old groups. On average per person 19.6 of teeth had attachment loss greater than 3mm. Among people with at least one side of attachment loss greater than 3mm on an average 36.6% of the teeth per person was affected.

**German dentist Adolph Witzel (1847-1906[34])** was the first individual to identify bacteria as the cause of periodontal disease.

**Willoughby D.Miller (1853-1907[34])** in his book "The Microorganisms of the human Mouth" published in 1890, described the features of periodontal diseases and he believed that the disease was not caused by a specific bacterium but by a complex array of various bacteria. This constitutes what was later known as the non specific plaque hypothesis.

In mid 1960s the classic studies of **Loe[55] et al and Theilade et al** convincingly demonstrated that plaque accumulation directly preceded and initiated gingivitis and led to the eventual destruction of periodontal tissues.

**Tanner et al in 1979[85]** demonstrated that the microbial composition of subgingival plaque taken from diseases sites differed substantially from the samples taken from healthy sites in chronic periodontitis.

**Haffajee & Socransky in 1994[36]** stated, that the periodontal diseases appear to be caused by a relatively definite group of periodontal pathogens acting alone or in combination and it is well established that periodontal diseases represent mixed infections of periodontal tissues caused primarily by anaerobic gram negative bacteria.

In **1998 Socransky et al[83]** examined over 13,000 subgingival plaque samples from 185 adult subjects, to demonstrate the presence of specific microbial group within the dental plaque. Six closely associated groups within dental plaque were recognized in which *T.forsythia, P.gingivalis & T.denticola* recognized as red complex.

The World Workshop in Periodontology (**consensus report 1996**)[47] designated *A.actinomycetemcomitans*, *P.gingivalis* and *T.forsythia* as periodontal pathogens.

***A.actinomycetemcomitans* and Periodontitis**

**Newman et al in 1976**[65] reported that, *A.actinomycetemcomitans* was first recognized as possible pathogen by its increased frequency of detection and higher numbers in lesions.

**Baehni et al in1979**[5] found that, *A.actinomycetemcomitans* produces a number of potentially damaging metabolites including a leukotoxin.

**Zambon J J et al in 1983**[93] stated, *A.actinomycetemcomitans* Serotype a was more commonly detected in samples from chronic periodontitis subjects.

**Rodenburg J P et al in 1990**[74] found that, *A.actinomycetemcomitans* has also been implicated in adult forms of destructive periodontal disease, but its role is less clear. The species has been isolated from chronic periodontitis lesions, but less frequently and in lower numbers than lesion in LAP subjects.

**Vanwinkelhoff et al in 1992**[91] treated 50 adults with severe generalized periodontitis and 40 subjects with refractory periodontitis who were cultured positive for *A.actinomycetemcomitans* using mechanical debridement and systemically administered amoxicillin and metronidazole, only one of subjects was culture positive for *A.actinomycetemcomitans* 3-9 months post therapy.

**Kolenbrander et al in 2000**[50] stated that, *A.actinomycetemcomitans* may attach to other colonizing bacterial species by co aggregation

**Rudney et al in 2001**[76] found that, at some point these organisms may move from the supra gingival to subgingival environment from this vantage point they may

then attach to and invade the epithelial lining of periodontal pocket and possibly penetrate the underlining connective tissues.

## *P.gingivalis* and periodontitis

*P.gingivalis* is a second consensus periodontal pathogen that continues to be intensely investigated organisms of this group form brown to black colonies on blood agar plates and were initially grouped into a single species,*B.meleninogenicus (BacteriumMelaninogenicum)*. **Burdon et al in 1928**[16] found that this black pigmented bacteriodes have a long history of association with periodontal diseases.

**Dzink et al in 1988**[25] found that, *Porphyomonas gingivalis* has been found in higher numbers on or in epithelial cells recovered from the periodontal pocket then in associated plaque.

**Bragd et al in 1987**[11] reported that, *P.gingivalis* has been shown to be reduced in successfully treated sites but was commonly encountered in sites that exhibited reoccurrence of diseases or persistence of deep periodontal pockets post therapy.

**Holt et al in 1988**[44] demonstrated that *P.gingivalis* suppressed by systemic administration of rifampin but the re introduction of *P.gingivalis* resulted in initiation and progress of the lesion.

**Beck et al in 1990**[9] stated that *P.gingivalis* has been associated with an increased risk of periodontal disease severity and progression.

**Haffajee and socransky in 1994**[36] observed that, studies initiated in the late 1970s and extending to the present have strengthened the association of *P.gingivalis*

with disease and demonstrated that the species is uncommon and in low numbers in health or gingivitis but more frequently detected in destructive forms of disease.

***Tannerella forsythia* and Peridontitis**

*Tannerella forsythia* was the third consensus periodontal pathogen, and it was first described in 1979 as a "fusiform" Bacteroides. **Tanner et al in 1979[85]**

**Lai et al in 1987[52]** reported findings using fluorescent labeled polyclonal antisera and demonstrated that *T.forsythia* was much higher in subgingival than supra gingival plaque samples.

Initially *T.forsythia* was thought to be a relatively uncommon subgingival species. However the studies of **G Mur R et al in 1989[30]** using mono clonal antibodies to enumerate the species directly in plaque samples suggested that the species was more common than the previously found in culture studies and its levels were strongly related in increased pocket depth.

**Haffajee et al in 1997[37]** observed that, *T.forsythia* has been shown to be decreased in frequency of detection and counts after successful periodontal therapy including scaling and root planing.

**Vanwinkelhoff et al in 2002[90]** found that, *T.forsythia* was in much higher counts, proportions and prevalence in subjects with various forms of periodontitis than in periodontally healthy subjects.

**Hamlet et al in 2004**[38] showed that, there was a greater risk of periodontal attachment loss in adolescents who were colonized by *T.forsythia* than adolescents in whom the species was not detected.

## *T.denticola* and periodontitis

**Keyes&Rams in 1983**[48]observed that, the Spirochetes have been considered as possible periodontal pathogens since the late 1800's and in the 1980s enjoyed the resurgence of interest for use as possible diagnostic indicators of disease activity and for therapeutic efficacy.

**Simonson L.G et al in 1988**[81] showed that , *T.denticola* was found to be more common in periodontally diseased than healthy sites, more common in subgingival than supra gingival plaque.

**Simonson L.G et al in 1992**[82] found that, *T.denticola* was shown to decrease in successfully treated periodontal sites, but not change or increase in non responding sites.

**Kigure et al in 1995**[49] observed that, *T. denticola* have been shown to be at the fore front of periodontal lesions as demonstrated in sections of undisturbed subgingival plaque using immuno histochemical localization.

**Riviere et al in 1997**[71] showed their presence in periodontally healthy sites was related to an increased risk of development of periodontitis. The reduction is so consistent that it has been used in some studies as a measure of compliance in determining whether subjects used the prescribed antibiotics.

## PERIODONTITIS AND ATHEROSCLEROSIS

The concept of systemic diseases originating in dental and oral infections had been mentioned in Assyrian Clay tablets by **Hippocrates(460-370BC)**[17].

In the 19th Century, **Benjamin Rush in 1818**[17] **and Leonard Koecker in 1828**[17] recognized the role of oral sepsis in rheumatic and other diseases.

Later in the 19th Century **W.D.Miller in 1891**[17] also mentioned oral infections as the cause of many diseases.

**William Hunter (1861-1937)**[45] **in 1900**, a British physician indicated dentistry as being the cause of oral sepsis which in turn caused rheumatic and other chronic diseases.

A number of studies have reported associations between periodontitis and heart disease. Probably the earliest study was published in **1963 by Mackenzie and Millerd)**[17]. The study was conducted onto individuals with suspected diabetes and 54 atherosclerotic individuals. The only group to exhibit more bone loss than it control was the atherosclerotic non diabetic group in which 63% had more bone loss than controls.

**Silver et al in 1977**[80] stated frequent bacteraemia occur as a result of daily activities such as tooth brushing or chewing in periodontitis patients.

**Nery E.B. et al in 1987**[64] in their article titled "Prevalence of Medical problem in periodontal patients obtained from three different populations" reported that cardiovascular disease was the most prevalent problem in all groups.

**Mattila and colleagues in1989**[59] were the first to show a statistical association between dental infections and advanced atherosclerosis.

**In 1993, Destefeno and colleagues**[23] examined the relationship between periodontal disease and CHD and observed an approximately 25% increased risk for CHD (RR 1.25, CI 1.06 – 1.48)individuals with periodontal disease after controlling for race, education, systolic blood pressure, cholesterol, alcohol consumption, Body Mass Index (BMI), exercise and poverty index. This was the first large scale study with a follow up period of 14 years and adjusting for confounding factors.

**Genco et al in 1997**[32] studied the relationship between tooth loss and alveolar bone loss and CVD as confirmed by Electro Cardio Gram (ECG). They observed a high relative risk of 2.69 for CVD with alveolar bone loss.

**Noack et al in 2001**[66] observed statistically significant increase in CRP levels in 109 subjects with moderate to severe periodontitis when compared with 65 healthy controls. After adjustments for factors known to be associated with elevated CRP, including age, smoking, BMI, triglycerides and cholesterol subjects with high levels of Mean clinical attachment loss had significantly higher CRP levels (4.06 ± 5.55mg/L) than did controls (1.70 ± 1.91 mg/L, P=0.011)

**Bhulin et al in 2003**[13] reported that the presence of periodontal pathogens-including *Porphyromonasgingivalis, Prevotella intermedia* and *T.forsythia*- from subgingival plaque samples was positively associated with elevated CRP levels

(p=0.029).CRP levels were also reported to be higher in 50 CVD patients with severe periodontitis ≥ 4mmPD.

**Chiu B in 1999**[18] isolated *P.gingivalis and S.sanguis* in the arterial plaque from a carotid endarterectomy specimen that suggested possible invasion of the periodontopathogens in the carotid atheroma.

**Dorn and colleagues in 1999**[24] reported that *P.gingivalis* appeared to invade coronary artery endothelial cells and increase the degradation of endothelial cell proteins.

**Haraszthy and colleagues in 2000**[39] using specific oligonucleotide primers in polymerase chain reaction (PCR) assays , detected microbial ribosomal RNA in atheromatous plaque .They were *C.pneumoniae* ,as well as dental pathogen and Cyto Megalo Virus .30% were positive for *T.forsythia*, 26% for *P.gingivalis* and 18% for *A.actinomycetem comitans* and 14% for *P intermedia*.

**Choi and colleagues in 2002**[19] isolated P.gingivalis heat shock protein ,T – specific T-cells in atherosclerosis plaque from subjects with severe atherosclerosis.

**Taylor- Robinson and colleagues in 2002**[87] isolated several infectious agents by DNA identification methods from all major arteries affected by atherosclerosis. Nearly 40% of specimens were positive for *Chlamydia pneumoniae* and 35.4% were positive for mixture of *Chlamydia* and *A.actinomycetemcomitans* and *P.intermedia*.These findings suggest a possible invasion of the major arteries by oro-dental pathogens.

**Padilla C et al in 2006**[67] stated that, the presence of *A .actinomycetem comitans* in atheromatous plaques and the periodontal pockets of the same patients could indicate a role for periodontal pathogenic bacteria in the atherosclerosis disease process.

**Pucar A et al in 2007**[70]**,** stated the absence of putative pathogenic bacteria in internal mammary arteries, which are known to be affected rarely by atherosclerotic changes, and their presence in a high percentage of atherosclerotic coronary arteries support the concept that periodontal organisms are associated with the development and progression of atherosclerosis

**Almetti M et al in 2007**[1] observed that, bacterial DNA was detected in 31 out of 33 endarterectomy specimens. However, none of the samples tested positive for DNA from periodontal pathogens. The presence of periodontal bacteria in atheromatous plaques was not confirmed by this investigation, thus no correlation could be drawn between periodontitis bacteria and microorganisms involved in the atherosclerotic lesions.

**Mustapha T Z et al in 2007**[63] reported that, periodontal disease with elevated bacterial exposure is associated with CHD events and early atherogenesis, suggesting that level of systemic bacterial exposure from periodontitis is the biologically pertinent exposure with regard to atherosclerosis risk.

**Rufail M L et al in 2007**[77] found that, the periodontal infection is associated with elevated plasma levels of atherogenic lipoprotein species. This association may account for the increased risk of periodontitis patients for cardiovascular disease.

**Elkam R et al in 2008**[26] found that, the presence of periodontal pathogens in atherosclerotic plaques and in apparently healthy vessels appeared to reflect a higher level of bacteraemia rather than infection of endothelial cells.

**Monterio A M et al in 2009**[61] studied the cardiovascular disease parameters in periodontitis patients. The level of triglycerides and high density lipoprotein in periodontitis patients were significantly higher and lower, respectively compared to controls. total cholesterol, low- density lipoprotein and lipid peroxide level were the same in both groups. Interleukin IL -6 and -8, antibodies against oxidized low – density lipoprotein, and leukocyte and neutrophils count were significantly higher in periodontitis patients. The results confirmed and further strengthened the suggested association between coronary artery disease and periodontitis.

**Buhlin K in 2009**[14] in his study showed increased levels of antibodies against Hsp 65 and 70 and decreased level of antibodies against Hsp 60 periodontitis was associated with increased levels of CRP, glucose, fibrinogen and IL -18 and with decreased levels of IL-4.

**Buhlin K in 2009**[15] in his study investigated the effect of mechanical infection control for periodontitis on the prevalence of well established risk factors for atherosclerosis. 12 months after periodontal treatment HDL-concentration were increased where as LDL-concentration decreased haptoglobin concentration were also lowered and IL18 and INF- $\gamma$ level lowered but no effect on plasma levels of IgA, IgG1 and IgG2 antibodies against heatshock proteins. This study indicates that standard treatment for periodontal disease induces systemic changes in several biochemical markers that reflect the risk of atherosclerosis.

**Tonetti MS in 2009**[89] stated that, the Periodontitis has effects that go beyond the oral cavity and its treatment and prevention may contribute to the prevention of atherosclerosis.

# MATERIALS AND METHOD

## SUBJECT SELECTION

25 subjects who underwent endarterectomy were selected and screened, out of the patients who hospitalized at the coronary care unit, Frontier life line Hospital, DR.K.M.Cherian Heart Foundation,Chennai – 600101. The subjects were examined periodontally, subgingival plaque samples, and their respective carotid atheromatous plaques were collected.

## INCLUSION CRITERIA

- Patients underwent endarterectomy
- Either sex
- Age 40-70 yrs
- At least 14 teeth should be present
- At least 3 teeth with CAL≥ 4 mm

## EXCLUSION CRITERIA

- Edentulous patients
- Critically ill cardiac patients
- All third molar teeth are excluded

## STUDY PROTOCOL

- Medical history and informed consent
- Periodontal examination using clinical parameters namely plaque index, probing pocket depth and clinical attachment level.
- Collection of subgingival plaque sample
- Collection of atheromatous plaque sample following endarterectomy
- DNA isolation
- Polymerase chain reaction for amplification of **16S rDNA *Porphyromonas gingivalis, Treponema denticola, Aggregatibacter actinomycetemcomitans,Tannerella forsythia.***
- Gel electrophoresis
- Sequencing of the amplified product
- Statistical analysis

Following the approval from Institutional Ethical Committee, subjects were selected, informed consent was obtained from all the subjects selected for the study after explaining the study procedure. Prior to the examination a thorough medical and dental history was taken.

Intra-oral examination was done using mouth mirror and William's periodontal probe. Periodontal evaluation was done by measuring the plaque index, probing pocket depth (PPD) and clinical attachment level (CAL). Atheromatous plaque sample of the corresponding patients were received from the histopathology department.

## CLINICAL PARAMETERS

### Plaque Index (Silness & Loe'1964[17])

Teeth examined - All teeth

Surfaces examined - Distofacial, facial, mesiofacial, lingual.

**Criteria for Scoring**

Score 0: No Plaque

Score 1: Film of plaque at the marginal area recognized by running of a probe

Score 2: Moderate accumulation that can be seen by naked eye

Score 3: Abundance of soft deposits

$$\text{Plaque index per teeth} = \frac{\text{Total Score}}{4}$$

$$\text{Plaque index per individual} = \frac{\text{Total PI per tooth}}{\text{Total number of teeth examined}}$$

**Interpretation**

0 - Excellent

0.1-0.9 - Good

1.0-1.9 - Fair

2.0-3.0 – Poor

## PROBING POCKET DEPTH (PPD) (GRANT 1965[35])

Probing pocket depth is measured from the gingival margin to the base of the pocket using William's periodontal probe. The probe is passed under the gingiva along the circumference of the tooth. Three measurements are made on the buccal aspect and three on the lingual aspect of each tooth-total of six sites per tooth.

## CLINICAL ATTACHMENT LEVEL (CAL) [Carranza AF[17]]

Clinical attachment level is measured from the cementoenamel junction to the base of the pocket using William's periodontal probe. When the gingival margin is located on the anatomic crown, the level of the attachment is determined by subtracting from the probing pocket depth, the distance from the gingival margin to the cementoenamel junction.

If both are the same, the loss of attachment is zero. When the gingival margin coincides with the cementoenamel junction, the loss of attachment equals the probing pocket depth.

When the gingival margin is located apical to the cementoenamel junction, the loss of attachment is greater than the probing pocket depth and therefore the distance between the cementoenamel junction and the gingival margin should be added to the probing pocket depth.

Three measurements are made on the buccal aspect and three on the lingual aspect of each tooth-total of six sites per tooth.

## COLLECTION OF SUBGINGIVAL PLAQUE SAMPLE

For each patient, the deepest pocket were selected and isolated for the sample collection and subgingival plaque samples were taken from the bottom of periodontal pockets from the deepest site with a sterile Gracey curette after removal of the supra gingival plaque.

The plaque is dislodged from the curette by gentle rotation in a labeled vial containing 500 µl of sterile phosphate buffered saline. (PBS) (pH 7.8) and kept in a thermos ice pack, the samples were transferred to the laboratory (IBMS, Tharamani, Chennai) stored at $-20^0$ C till the time of Microbiological analysis.

## DNA EXTRACTION:

The samples obtained were centrifuged and supernatant was discarded. To the residual 200 µl of lysis buffer was added, vortexed and it was boiled for 10 minutes and then micro centrifuged at 10,000 rpm for 3 minutes. The resultant supernatant was stored at -20 ºC till the assay. And 10 µl of the supernatant was directly used as template in PCR.

## COLLECTION OF ATHEROMATOUS PLAQUE SAMPLE

Atheromatous plaque sample collected in a labelled vial containing 500 µl of sterile phosphate buffered saline (PBS) (pH 7.8) during endarterectomy was received from histopathology department. And kept in a thermos ice pack, the samples were transferred to the laboratory (IBMS, Tharamani, Chennai) stored at $-20^0$ C till the time of Microbiological analysis.

## DNA EXTRACTION:

The atheromatous Plaque were dissociated with a spatula and then homogenized thoroughly with a tube pestle. The samples obtained were centrifuged and supernatant was discarded. To the residual 200 µl of lysis buffer was added, vortexed and it was boiled for 10 minutes and then micro centrifuged at 10,000 rpm for 3 minutes. The resultant supernatant was stored at -20 ºC till the assay. And 10 µl of the supernatant was directly used as template in PCR.

## COMPOSITION OF LYSIS BUFFER

- 10 mM Tris-HCl
- 1.0mM EDTA
- 1.0 % Triton X-100 (pH 7.8)

## POLYMERASE CHAIN REACTION

The PCR involves the enzymatic amplification of DNA in vitro. This method is capable of increasing the amount of the target DNA sequence in a sample by synthesizing many copies of the DNA segment.

PCR is carried out in discrete cycles and each cycle of amplification can, (if 100 % efficient) doubles the amount of target DNA. The target DNA is exponentially amplified such that after n cycles, there is 2n times as much target DNA as was present initially **(Erlich HA et al 1991[28])**.

The basic technique of PCR includes repeated cycles of amplifying selected nucleic acid sequences (**Mullis KB et al 1987[62]).**

Each cycle consists of three steps

1. A DNA denaturation step, in which the double strands of the target DNA are separated

2. A primer annealing step, performed at a lower temperature, in which primers anneal to their complementary target sequences

3. An extension reaction step, in which DNA polymerase extends the sequences between the primers.

## PRIMERS UTILIZED IN THIS STUDY

| SNO | PRIMER NAME | PRIMER SEQUENCE | FRAGMENT SIZE |
|---|---|---|---|
| 1 | 16S rDNA forward *A.actinomycetemcomitans*<br>16S rDNA reverse *A.actinomycetemcomitans* | 5'-AAA CCC ATC TCT GAG TTC TTC TTC-3'<br>5'-ATG CCA ACT TGA CGT TAA AT-3' | 557bp |
| 2 | 16S rDNA forward *P.gingivalis*<br>16S rDNA reverse *P.gingivalis* | 5'-AGG CAG CTT GCC ATA CTG CG-3'<br>5'-ACT GTT AGC AAC TAC CGA TGT -3' | 404bp |
| 3 | 16S rDNA forward *T.denticola*<br>16S rDNA reverse *T.denticola* | 5'-TAA TAC CGA ATC TGC TCA TTT ACA T-3'<br>5'-TCA AAG AAG CAT TCC CTC TTC TTC TTA -3' | 316bp |
| 4 | 16S rDNA forward *T.forsythia*<br>16S rDNA reverse *T.forsythia* | 5'-GCG TAT GTA ACC TGC CCG CA -3'<br>5'-TGC TTC AGT GTC AGT TAT ACC T-3' | 641bp |

***Ashimoto et al oral microbiology and immunology 1996:11:266-273(2)***

**PREPARATION OF WORKING STOCK**

The primers were diluted from the stock as per the required concentrations. For one reaction, the PCR reaction mixture contains the following reagents.

| **REAGENT** | **Volume in µl** |
|---|---|
| 10 X PCR Buffer | 5.0 |
| dNTP 0.25Mm **(medox)** | 1.0 |
| Forward primer →25 picomol (sigma) | 1.0 |
| Reverse primer →25 picomol (sigma) | 1.0 |
| $Mgcl_2$ -2.5mmol | 1.0 |
| Taq polymerase (banglore genei)(2 units) | 0.5 |
| Template | 10.0 |
| Sterile milipore water | 30.5 |
| Total reaction volume | 50 |

## PCR THERMOCYCLING PROGRAMME

90 µl of the PCR reaction mix was pipetted into micro centrifuge tubes.10 µl of the template DNA was added and mixed thoroughly. The micro centrifuge tubes were placed in a thermocycler (AB biosystem) and cycling conditions were set.

## THERMAL CYCLING CONDITIONS

1. Initial denaturation step at 94$^{o}$C for 5 minutes followed by
2. 35 cycles of
    - Denaturation at 94$^{o}$C for 1 minute
    - Primer Annealing at 50$^{o}$C for 1 minute
    - Extension at 72$^{o}$C for 1.5 minutes and
3. Final extension step at 72$^{o}$C for 7 minutes

## GEL ELECTROPHORESIS FOR DETECTION OF AMPLICON

The PCR product was detected by 1.5% Agarose gel electrophoresis.

## REAGENTS REQUIRED

1. Preparation of TAE Buffer (1x)
    - 490 ml of double distilled water
    - 10 ml of 50 x TAE Buffer
2. Ethidium bromide
    - Ethidium bromide - 10 mg
    - Distilled water - 1 ml

## PROCEDURE

## PREPARATION OF 1.5% AGAROSE GEL

- 1.5 gram of agarose weighed and transferred into 250 ml conical flask.
- 100 ml of 1x TAE buffer was added to it, mixed gently and boiled in a microwave oven.
- The appropriate sized gel tray and comb was washed. Cello tape was fixed on both sides of the tray. The comb was placed on the gel tray without touching the bottom and left on an even surface.
- Agarose was cooled down, 0.5 µl of ethidium bromide was added and mixed well. It was poured on the gel tray and allowed to polymerize.

## PREPARATION OF SAMPLE AND LOADING

- The cello tape was removed from the gel tray and the tray placed in the electrophoresis tank.
- 1x TAE buffer was poured into the tank until the gel gets immersed.
- The comb was carefully removed from the gel tray.
- 10 µl of PCR product was mixed with 10 µl of 2x gel loading buffer and loaded into the wells
- The electrodes were connected.
- The power was switched ON and set at 100 V.
- After the completion of the electrophoresis, gel was taken to the transilluminator and observed under UV-light. (Biorad gel documentation)

**INTERPRETATION:**

100 bp DNA ladder **(MEDOX)** was used as a size marker and sterile milli pore water was used as blank control.

**SEQUENCE OF PCR PRODUCT**

The PCR product of 404 bp of 16S rDNA of *porphyromonas gingivalis,* 557 bp of 16S rDNA of *Aggregatibacter actinomycetemcomitans*, 641 bp of 16S rDNA of *Tannerella forsythia,* 316bp of 16S rDNA of *Treponema denticola* positive samples were given to **MWG – BIOTECH, GERMANY** for sequencing the PCR products by automated DNA sequencer.

The data was collected and statistically analyzed.

# ARMAMENTARIUM

## CLINICAL EXAMINATION AND SAMPLE COLLECTION

- Mouth Mirror
- William's periodontal probe
- Tweezer
- Face Mask
- Head cap
- Surgical gloves
- Cotton rolls
- Sterile Gracey curettes
- Storage vials with phosphate buffered saline(500µl)
- Thermos box with gel pack

## DNA ISOLATION AND PURIFICATION

- Eppendorf tubes
- -20$^{o}$C freezer
- Micro centrifuge
- Water bath
- Micropipette
- Micro pipette tips

## POLYMERASE CHAIN REACTION

- PCR tubes
- Micropipette

- Micro centrifuge
- PCR thermal cycler
- Eppendorf tube
- Primers
- Sterile Millipore water

## AGAROSE GEL ELECTROPHORESIS

- Gel tray
- Gel comb
- Cello tape
- Agarose
- 10x TAE buffer
- Ethidium bromide
- Electronic weighing balance
- Electrophoresis tank with power supply
- UV trans illuminator
- Microwave oven

# STATISTICAL ANALYSIS

Proportions of different organisms were estimated from the subgingival samples and atheromatous plaque samples.

McNemar's Test (1 tailed) was used to calculate the overall p value.

McNemar's Test was used to compare the positive rates of *P.gingivalis, T .denticola, T.forsythia* in samples from both subgingival and atheromatous plaque.

**STATCALC** procedure available in **SPSS version 13** was used for all statistical analysis.

In the present study, **< 0.05** was considered as the level of significance.

**McNemar's Test**

The formula used is

$$\chi^2 = [(|b-c|-1)^2]/(b+c)$$

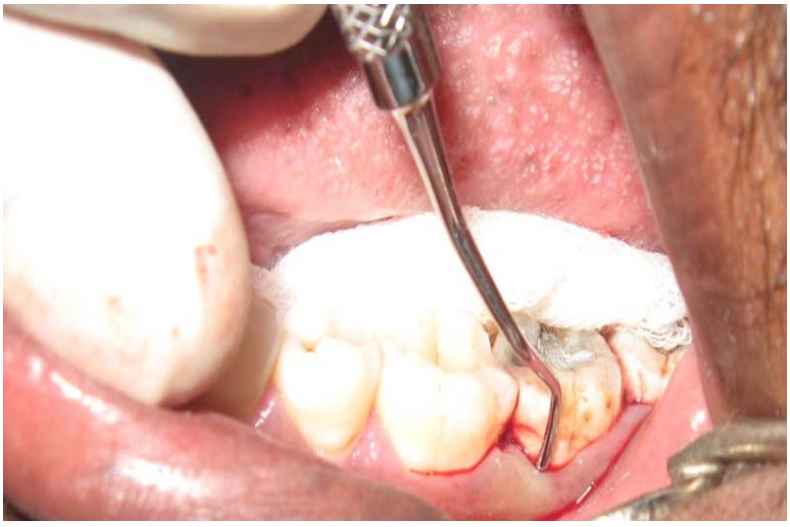
where b and c are the discordant cell frequencies in the 2x2 matrix.

$\chi^2$ follows a chi-square distribution with one degree of freedom.

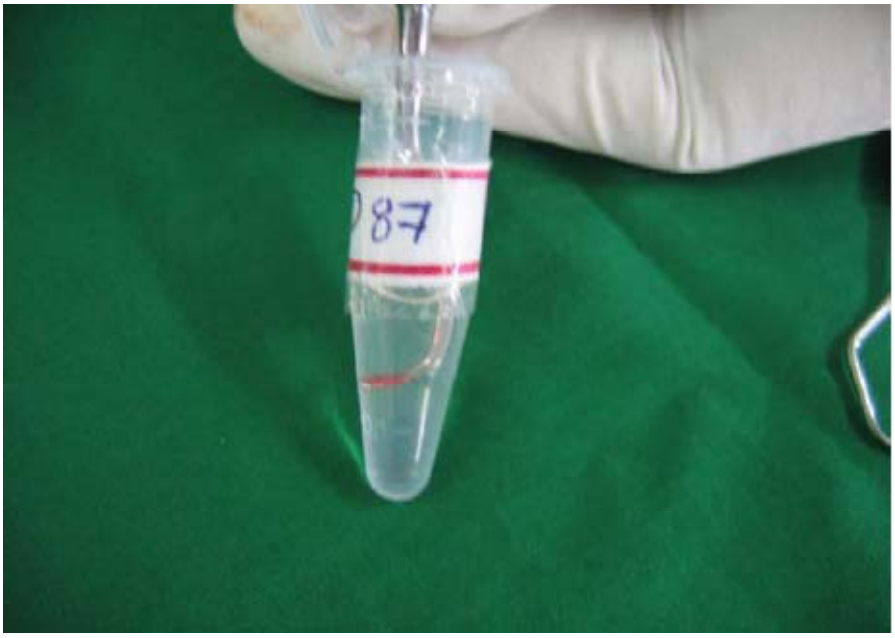

**Photograph No.1 Armamentarium for Clinical Examination and sample collection**

**Photograph No.2 Chronic Periodontitis**

**Photograph No.3 examination of Sample Site**



**Photograph No.4 Insertion of Curette**



**Photograph No.5 Sub Ginvial Plaque Sample**

**Photograph No.6 Atheromatous Plaque Samples**


**Photograph No.7 Sample Transport**

**Photograph No.8 Water Bath – Front View**

**Photograph No.9 Water Bath – Inner Aspect**

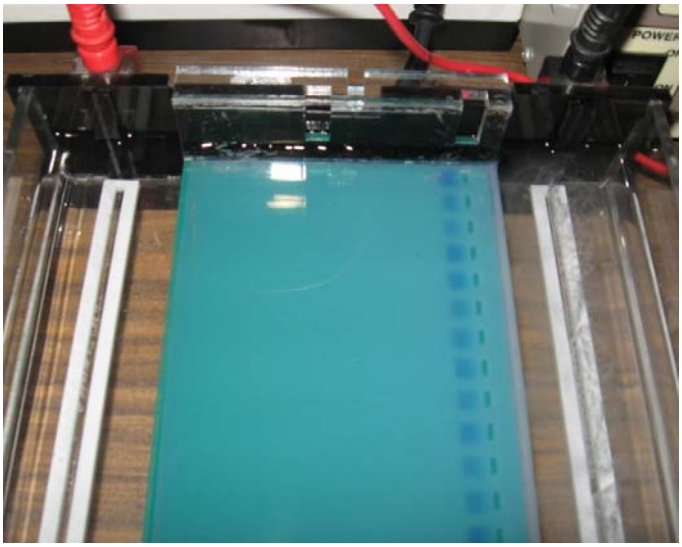

**Photograph No.10 Micro Centrifuge**

**Photograph No.11 Reagents for PCR**

**Photograph No.12 Armamentarium for PCR**


**Photograph No.13 Thermal Cycler**


**Photograph No.14 Armamentarium for Gel Electrophoresis**



**Photograph No.15 Gel Electrophoretic Apparatus**

**Photograph No.16 UVBiorad Gel Documentation System**

# RESULTS

Twenty five patients who underwent endarterectomy were selected and screened at the coronary care unit, Frontier Lifeline Hospital, DR.K.M.Cherian Heart Foundation,Chennai – 600101. The subjects were examined periodontally, and subgingival plaque samples were collected from the deepest pocket and their atheromatous plaque samples were obtained from histopathology department of Frontier Lifeline Hospital.

The clinical parameters used were probing pocket depth, clinical attachment loss, CPK, CPK-MB and LDH.

The subgingival plaque samples and atheromatous plaque samples were examined with PCR technique using specific primers for periodontal bacteria. DNA sequences of the amplified products of *Tannerella forsythia*, *Treponema denticola*, and *porphyromonas gingivalis* were compared with type strains sequences from the GenBank by blast to confirm their sequence homology.

Table 1 summarises the characteristics of cases included in the study, such as number of patients, age, gender, probing pocket depth, clinical attachment loss, and the site of subgingival samples collection.

Table 2 summarises the characteristics of cases included in the study, such as number of patients, age, gender, CPK, CPK-MB, LDH and the site of atheromatous plaques samples collection.

Table 3 and Figure 1 shows the prevalence of P.gingivalis microorganisms in subgingival plaque samples and atheromatous plaque samples.

Table 4 and Figure 2 shows the prevalence of T.forsythia microorganisms in subgingival plaque samples and atheromatous plaque samples.

Table 5 and Figure 3 shows the prevalence of T.denticola microorganisms in subgingival plaque samples and atheromatous plaque samples.

Table 6 and Figure 4 shows the prevalence of A.actinomycetemcomitans microorganisms in subgingival plaque samples and atheromatous plaque samples.

Table 7 shows the comparative prevalence of P.gingivalis microorganisms in both subgingival and atheromatous plaque samples.

Table 8 shows the comparative prevalence of T.forsythia microorganisms at subgingival plaque samples and atheromatous plaque samples.

Table 9 shows the comparative prevalence of T.denticola microorganisms at subgingival plaque samples and atheromatous plaque samples.

The DNA of atleast one of the target bacteria was detected in 96% of the subgingival sample. The prevalence of *P.gingivalis, T.denticola,T.forsythia* was 92% ,80%, and 96% respectively. The sequence of periodontal bacterial DNA in the atheromatous plaques could be ascertained by DNA sequencing and they were compared with type strains sequences from the Gen Bank by blast to confirm their sequence homology. The prevalence of *P.gingivalis, T.forsythia, T.denticola* was 64%, 56% and 76% respectively. The DNA of periodontal bacteria except *A.actinomycetemcomitans* was detected in both subgingival samples and atheromatous samples.

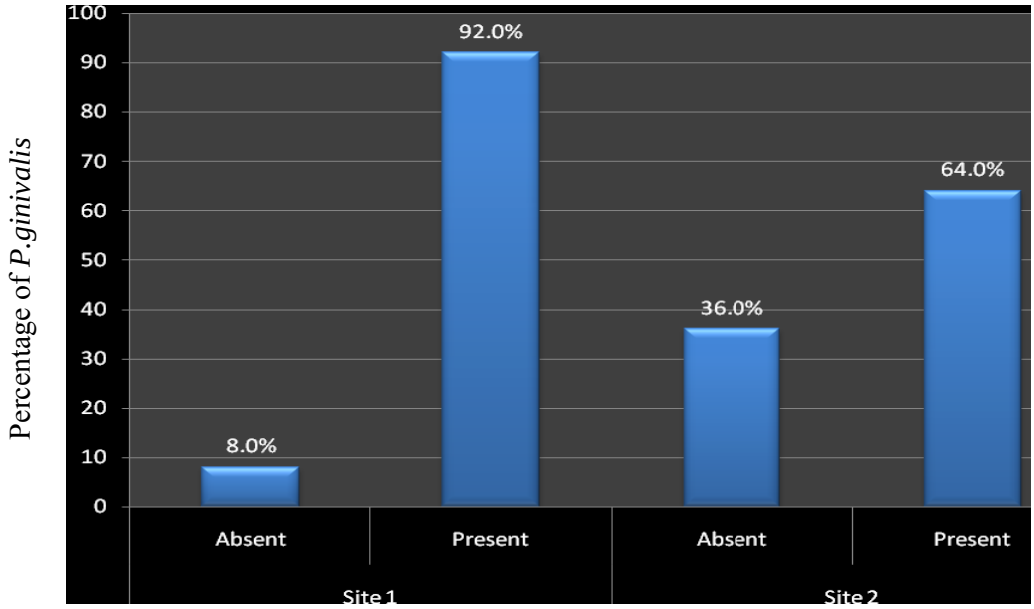
In 16 of 25 cases, *P.gingivalis* was present in both subgingival samples and atheromatous plaque samples at P value 0.008. In 19 cases, T.denticola was present in both subgingival samples and atheromatous plaque samples at P value 0.031 and T.forsythia were present in both subgingival and atheromatous plaque samples in 14 cases at P value 0.016.

**Table 3**

**PREVALENCE OF *P. gingivalis* MICROORGANISMS IN SUBGINGIVAL PLAQUE AND ATHEROMATOUS PLAQUE**

| ***P.gingivalis*** | **Subgingival plaque** | | **Atheromatous plaque** | |
|---|---|---|---|---|
| | **No.of cases** | **%** | **No.of cases** | **%** |
| Absent | 2 | 8.0 | 9 | 36.0 |
| Present | 23 | 92.0 | 16 | 64.0 |

**Figure: 1 PREVALENCE OF *P. gingivalis* MICROORGANISMS IN SUBGINGIVAL PLAQUE AND ATHEROMATOUS PLAQUE**




Site 1: SUBGINGIVAL PLAQUE

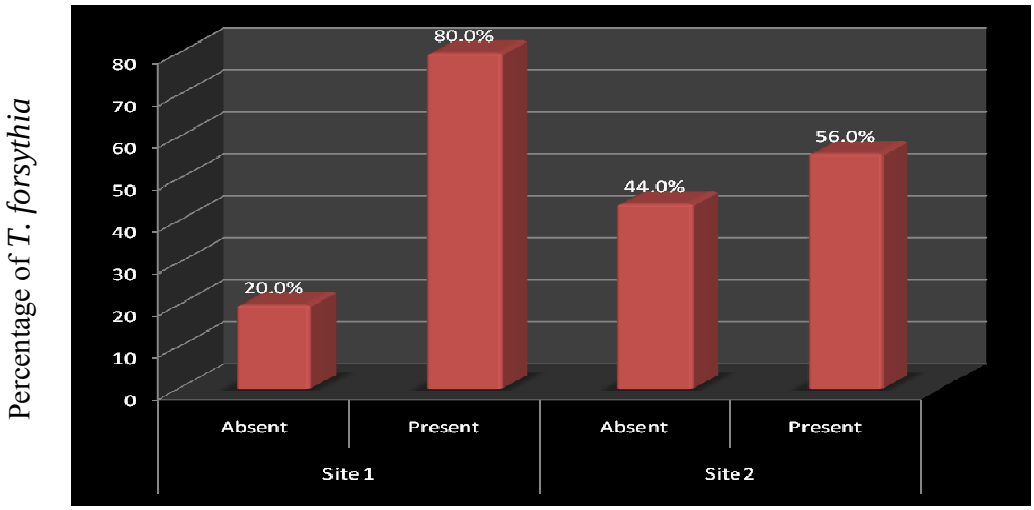
Site 2: ATHEROMATOUS PLAQUE

**Table 4**

**PREVALENCE OF *T. forsythia* MICROORGANISMS IN SUBGINGIVAL PLAQUE AND ATHEROMATOUS PLAQUE**

| *T.forsythia* | Subgingival plaque | | Atheromatous plaque | |
|---|---|---|---|---|
| | No of cases | % | No of cases | % |
| Absent | 5 | 20.0 | 11 | 44.0 |
| Present | 20 | 80.0 | 14 | 56.0 |

**Figure: 2 PREVALENCE OF *T. forsythia* MICROORGANISMS IN SUBGINGIVAL PLAQUE AND ATHEROMATOUS PLAQUE**




Site 1: SUBGINGIVAL PLAQUE

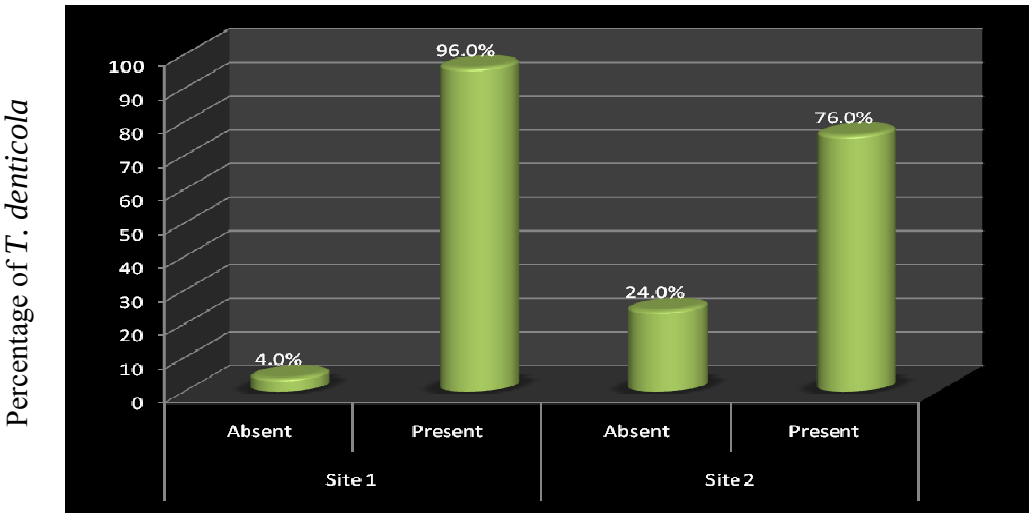
Site 2: ATHEROMATOUS PLAQUE

**Table 5**

**PREVALENCE OF *T. denticola* MICROORGANISMS IN SUBGINGIVAL PLAQUE AND ATHEROMATOUS PLAQUE**

| ***T.denticola*** | **Subgingival plaque** | | **Atheromatous plaque** | |
|---|---|---|---|---|
| | **No of cases** | **%** | **No of cases** | **%** |
| Absent | 1 | 4.0 | 6 | 24.0 |
| Present | 24 | 96.0 | 19 | 76.0 |

**Figure : 3 PREVALENCE OF *T. denticola* MICROORGANISMS IN SUBGINGIVAL PLAQUE AND ATHEROMATOUS PLAQUE**




Site 1: SUBGINGIVAL PLAQUE

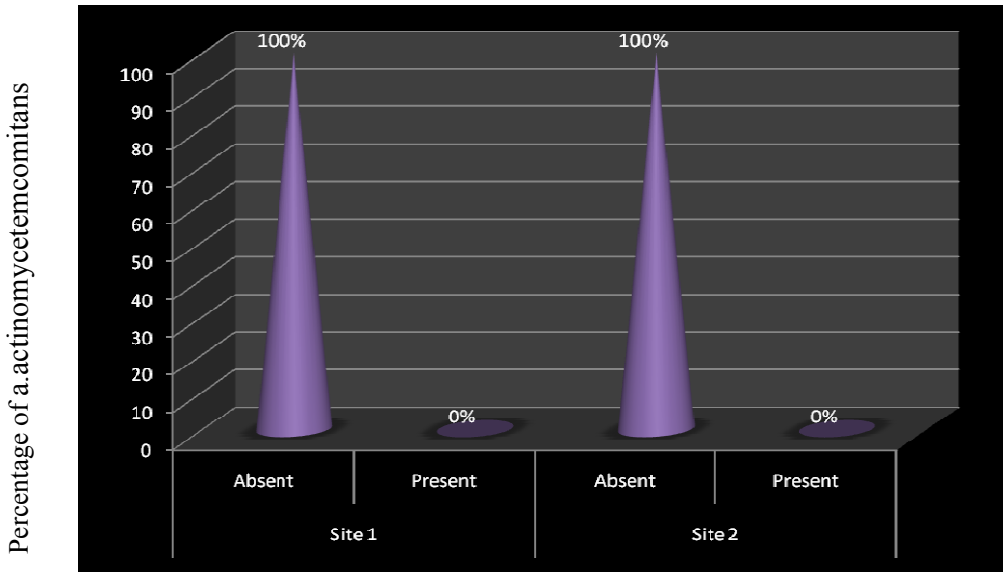
Site 2: ATHEROMATOUS PLAQUE

**Table 6**

**PREVALENCE OF *A.actinomycetemcomitans* MICROORGANISMS IN SUBGINGIVAL PLAQUE AND ATHEROMATOUS PLAQUE**

| A.actinomycetemcomitans | Subgingival plaque | | Atheromatous plaque | |
|---|---|---|---|---|
| | No. | % | No. | % |
| Absent | 25 | 100.0 | 25 | 100.0 |
| Present | 0 | 0.0 | 0 | 0.0 |

**Figure :4 PREVALENCE *OF A.ACTINOMYCETEMCOMITANS* MICROORGANISMS IN SUBGINGIVAL PLAQUE AND ATHEROMATOUS PLAQUE**




Site 1: SUBGINGIVAL PLAQUE

Site 2: ATHEROMATOUS PLAQUE

**Table 7**

**THE COMPARATIVE PREVALENCE OF *P.gingivalis* MICROORGANISMS IN SUBGINGIVAL PLAQUE SAMPLES AND ATHEROMATOUS PLAQUE SAMPLES.**

| | | Site 2 | | Total |
|---|---|---|---|---|
| | | **Absent** | **Present** | |
| Site 1 | Absent | 2 | 0 | 2 |
| | Present | 7 | 16 | 23 |
| Total | | 9 | 16 | 25 |
| % in both site 1and site2 | | | 64% | |
| P Value | | | 0.008 | |

Site 1: SUBGINGIVAL PLAQUE

Site 2: ATHEROMATOUS PLAQUE

**Table 8**

**THE COMPARATIVE PREVALENCE OF *T.forsythia* MICROORGANISMS IN SUBGINGIVAL PLAQUE SAMPLES AND ATHEROMATOUS PLAQUE SAMPLES.**

| | | Site 2 | | Total |
|---|---|---|---|---|
| | | **Absent** | **Present** | |
| Site 1 | Absent | 5 | 0 | 5 |
| | Present | 6 | 14 | 20 |
| Total | | 11 | 14 | 25 |
| % in both site 1 and site2 | | | 56% | |
| P value | | | 0.031 | |

Site 1: SUBGINGIVAL PLAQUE

Site 2: ATHEROMATOUS PLAQUE

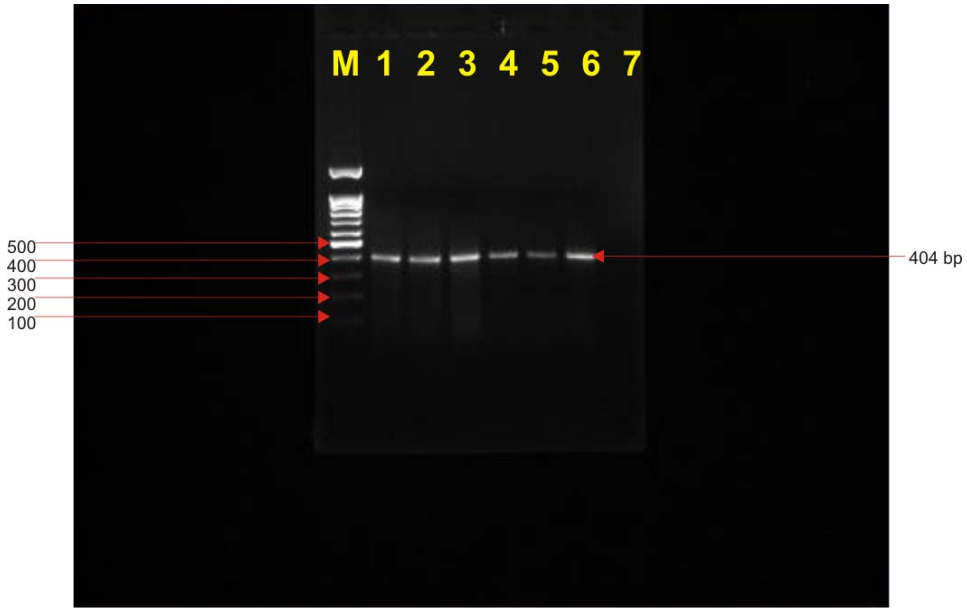
**Table 9**

**THE COMPARATIVE PREVALENCE OF *T.DENTICOLA* MICROORGANISMS IN SUBGINGIVAL PLAQUE SAMPLES AND ATHEROMATOUS PLAQUE SAMPLES.**

| | | Site 2 | | Total |
|---|---|---|---|---|
| | | Absent | Present | |
| Site 1 | Absent | 1 | 0 | 1 |
| | Present | 5 | 19 | 24 |
| Total | | 6 | 19 | 25 |
| | | | 76% | |
| P value | | | 0.016 | |

Site 1: SUBGINGIVAL PLAQUE

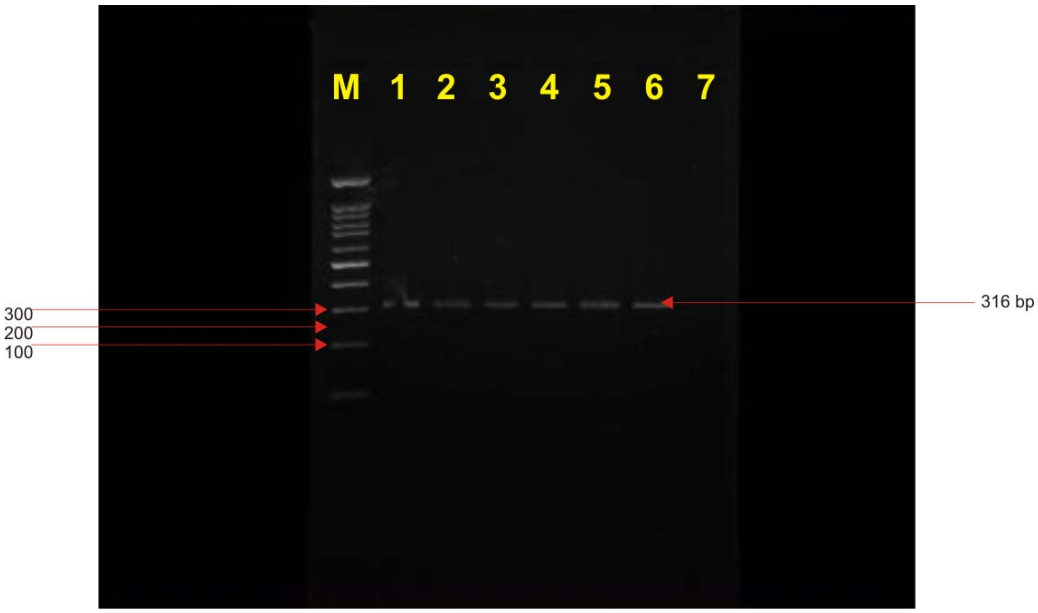
Site 2: ATHEROMATOUS PLAQUE




**Photograph No.17 Electrophoresis showing the amplified product of *P.gingivalis*.**

**M**-100 bp Ladder

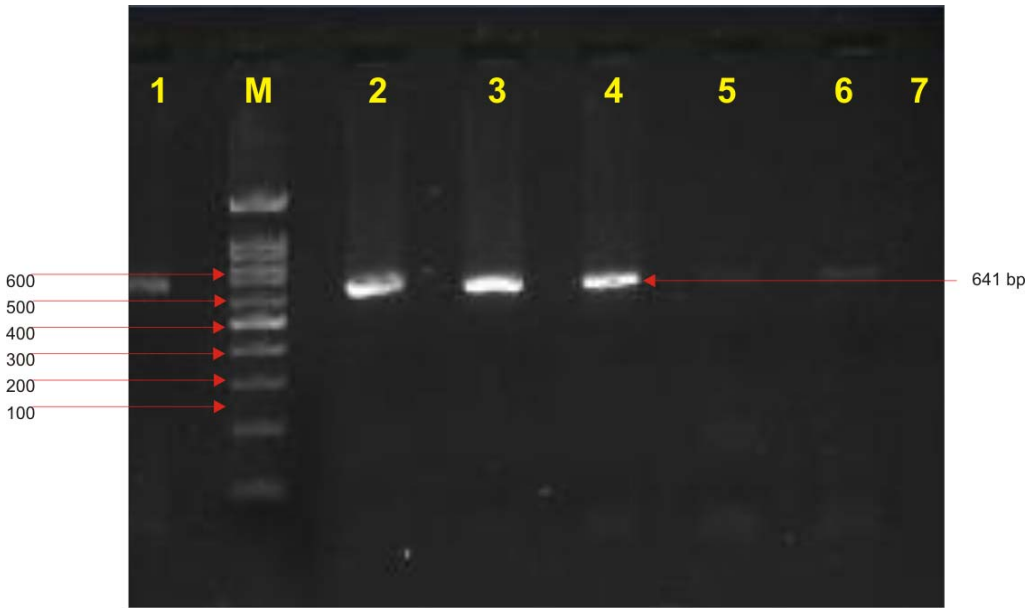
**Lane 1, 2, 3, 4, 5, 6** – *P.gingivalis* 16S rDNA (404 bp)
**Lane 7** – Blank Control




**Photograph No.18 Electrophoresis showing the amplified product of *T.denticola*.**

**M**-100 bp Ladder

**Lane 1, 2, 3, 4, 5, 6** – *T.denticola* 16S rDNA (316 bp)
**Lane 7** – Blank Control




**Photograph No.19 Electrophoresis showing the amplified product of *T.forsythia*.**

**M**-100 bp Ladder

**Lane 1, 3, 4, 5, 6** – *T.forsythia* 16S rDNA (641 bp)

**Lane 7** – Blank Control

# SEQUENCE OF PCR PRODUCT – INTERPRETATION

## SEQUENCE ANALYSIS OF 16S *rDNA* GENE of *P.GINGIVALIS*

The present study, the PCR positive amplicon of *P.gingivalis* positive sample of subgingival plaque and atheromatous plaque was purified and the nucleotide sequence was deduced by automated sequencer. The sequence was blasted in the blast programme available in the NCBI web site and found that the sequences aligned with the *P.gingivalis* nucleotide sequences available in the GenBank.

## SEQUENCE OF ANALYSIS OF 16S rDNA Gene SPECIFIC FOR PORPHYROMONAS GINGIVALIS (FORWARD PRIMER)

TGGTAGTCCACGCAGTAAACGATGATTACTAGGAGTTTGCGATATACCGTCAAGCTTCCA

CAGCGAAAGCGTTAAGTAATCCACCTGGGGAGTACGCCGGCAACGGTGAAACTCAAAGGA

ATTGACGGGGGCCCGCACAAGCGGAGGAACATGTGGTTTAATTCGATGATACGCGAGGAA

CCTTACCCGGGATTGAAATGTAGATGACGGATGGTGAAAACCGTCTTCCCTTCGGGGCGT

CTATGTAGGTGCTGCATGGTTGTCGTCAGCTCGTGCCGTGAGGTGTCGGCTTAAGTGCCA

TAACGAGCGCAACCCACATCG


Reverse

CCTTTCGGCCTTCTTCATTCACACGGCGTCGCTCCGTCAGACTTTCGTCCATTGCGGAAG

ATTCTTAGCTGCTGCCTCCCGTAGGAGTTTGGGCCGTATCTCAGTCCCAATGTGTCCGTT

CACCCTCTCAGGCCGGATACCCATCGTTGCCTTGGTGAGCCTTTACCTCACCAACCAGCT

AATGGGACGCGGGCCCATCCTGAAGCGGACCGTAGCTCCTTTCCTCATTTACCTTTATGT

AAA

**SEQUENCE ANALYSIS OF 16S *rDNA* GENE of *T.forsythia***

The present study, the PCR positive amplicon of *T.forsythia* positive sample of subgingival plaque and atheromatous plaque was purified and the nucleotide sequence was deduced by automated sequencer. The sequence was blasted in the blast programme available in the NCBI web site and found that the sequences aligned with the *T.forsythia* nucleotide sequences available in the GenBank.

**SEQUENCE OF *T.forsythia***

**Forward**

GGGGTTCCGCATGGGGCTATTTGTTAAGATTTATTGGTTGCGGATGGGCATGCGTACATT

AGGTAGTTGGTGAGGTACGGCTCACAAGCCATCGATGGTTAGGGGTTCTGAGAGGAAGGT

CCCCCACACTGG

**Reverse**

CATTCCGCCTACTTCATCTATACTCAAGACTACCAGTTTCAACGGCAATTTTA

**SEQUENCE ANALYSIS OF 16S *rDNA* GENE of** *T.denticola*

The present study, the PCR positive amplicon of *T.denticola* positive sample from subgingival plaque and atheromatous plaque was purified and the nucleotide sequence was deduced by automated sequencer. The sequence was blasted in the blast programme available in the NCBI web site and found that the sequences aligned with the *T.denticola* nucleotide sequences available in the GenBank.

**SEQUENCE OF** *Treponema denticola*

**Forward**

ATGAGGAAGGAGCTACGGCTCCGCTTCAGGATGGGCCCGCGTCCCATTAGCTGGTTGGTG

AGGTAAAGGCCCACCAAGGCAACGATGGGTATCCGGCCTGAGAGGGTGAACGGACACATT

GGGACTGAGATACGGCCCAAACTCCTACGGGGAGGCAGCAGC

## Reverse

CCTACATAGACGCCCCGAAGGGAAGACGGTTTTCACCATCCGTCATCTACATTTCAATCC

CGGGTAAGGTTCCTCGCGTATCATCGAATTAAACCACATGTTCCTCCGCTTGTGCGGGCC

CCCGTCAATTCCTTTGAGTTTCACCGTTGCCGGCGTACTCCCCAGGTGGATTACTTAACG

CTTTCGCTGTGGAAGCTTGACGGTATATCGCAAACTCCTAGTAATCATCGTTTACTGCGT

GGACTACCAGGGTATCTAATCCTGTTTGATACCCACGCCTTCGTGCTTCAGTGTCAGTCG

C

# DISCUSSION

Periodontitis is a chronic infectious disease characterized by inflammatory changes in the periodontal tissues. The disease occurs as a result of infection associated with a small number of predominately gram negative microorganisms.

Atherosclerosis has been defined as a progressive disease process that causes focal thickening of large to medium sized muscular and large elastic arteries.(**Beck JD et al 2000)**[8] Atherosclerosis is the most common cause of coronary artery disease (CAD) (**Becker AE et al 2001**)[10]. World Health Organisation statistics indicated that in 1993, cardiovascular diseases were responsible for 19% of deaths (10million) worldwide (**World health report 1995)**[92]**.**

In recent years, an increasing number of epidemiological studies have indicated that periodontitis may increase the risk of cardiovascular events. (**Beck JD et al 2001)**[7]. This effect may be due to the direct effect of periodontal pathogens or their product on endothelial cells via transient bacteraemia (**Geerts SO et al 2002**)[31]. In cell cultures, invasive *P.gingivalis* and fimbria stimulate endothelial cell activation, a necessary initial event in the development of atherogenesis (**Takahashi Y et al 2006)**[84]**.**

**Higashi et al in 2008**[43] reported that the periodontitis is associated with endothelial dysfunction in subjects without cardiovascular risk factors, as illustrated by a decrease in Nitrous Oxide bioavailability, and that systemic inflammation as a cause of endothelial dysfunction may lead to cardiovascular diseases.

A case control study showed that periodontitis is associated with endothelial dysfunction, and that 6 months after therapy, the benefits in oral health may be associated with improvement in endothelial function (**Tonetti MS et al 2007**)[88].

Experimental animal models (Apo E mice) demonstrated that P.gingivalis chronic inoculations increased lipid profiles,enhanced atheroma formation, and facilitated the calcification of atherosclerotic plaque (**Li L et al 2002)[54].**

**De stefeno et al in 1993[23]** based on 14 years of research on 9,760 individuals aged 25 to74 years reported an elevated risk of coronary disease in the group with periodontitis (25% higher).

**Beck et al in 1996[6]** recorded that the periodontal status of 1,147 individuals undergoing a 20 year long observation. In the group with advanced periodontal disease, there was an increased risk of severe cardiac episodes and cerebral stroke (1.9 and 2.8 times respectively) compared to the group without periodontitis.

**Finnish authors** have attempted to evaluate the relationship between chronic tooth –related infections, expressed as the number of lost teeth, and coronary disease. They showed that the incidence of coronary disease in individuals who had lost less than half of their teeth is 10% higher, rising to two –fold higher in the group that had lost more than half of their dentition. The number of teeth lost proved to be a significant risk factor of coronary disease, but only in smokers. **(Paunio K et al 1993)[69]**. It is difficult to evaluate the strength of association between periodontal and cardiovascular diseases, because those two conditions have several common risk factors (**ScannapiecoFA et al 2003**)[78].

However, recent randomized controlled clinical trials conducted by **D'Aiuto et al[21]** showed that intensive periodontitis therapy in otherwise generally healthy patients significantly reduces several inflammatory marker: IL6, cholesterol, and C-reactive protein levels and systolic blood pressure. According to the Framingham risk scale, intensive treatment of periodontitis reduces the relative probability of developing cardiovascular disease by 1.5% to 2% (**D'Aiuto F et al 2006)[21].**

It has been hypothesized that gram negative bacteria can migrate from pathogenic dental plaque to the blood stream, passing through the inflammatory, damaged epithelial attachment. The epithelial lining of the periodontal pocket, which frequently becomes thin and ulcerated in the course of periodontitis, may then provide an entry point for bacteria of subgingival plaque, allowing access to the underlying tissues and, eventually to the vasculature. Spreading with the blood, the bacteria can affect inflammatory cells present in atherosclerotic plaque and stimulate a cascade of processes leading to atherosclerotic plaque instability (**Erickson[27], Herzberg M.A[42], Haraszthy et al[39]).**

The present study does not unequivocally prove this theory, because bacteria were less frequent in atherosclerotic plaque than in dental plaque, and there was no distinct correlation between the amount of bacteria in subgingival plaque and coronary vessels.

On the other hand, our data do not exclude the bacterial theory, because *P. gingivalis*, *T.forsythia, and T.denticola* were found in the material collected from cardiac vessels; these bacteria are intimately related to chronic periodontitis.

Several methods have been applied to analyze a microbial population of subgingival plaque. The conventional method has been culture techniques. Because most putative periodontopathogenic bacteria are anaerobic and have fastidious growth behaviour, the selective medium used and the processing time required for culturing particular organisms, as well as the transport medium for samples, could contribute to the discrepancy in data analyzed. Species level identification of isolates is laborious and time consuming**.** [**Ali RW, et al 1994[3], Loesche WJ et al 1992[56]**] Immunological methods such as indirect immunofluorescence assay (IFA) or enzyme-linked immunosorbent assay (ELISA) require

Sensitivity ranges among the organisms have been reported as additional disadvantages for immunological methods. [**Melvin W. L et al 1994[60]**] The detection limit of whole chromosomal DNA probes is known to be about 104 cells whereas PCR can detect organisms of less than 100 cells.[**Ashimoto A et al 1996[2]**]Hence PCR technique was opted for this study.

PCR is carried out in discrete cycles and each cycle of amplification can, if 100 % efficient doubles the amount of target DNA. The target DNA is exponentially amplified such that after n cycles, there is 2n times as much target DNA as was present initially. [**Erlich HA et al 1991[28]**] The *16S rDNA* PCR proved to be an accurate, relatively straightforward, and reproducible method for identification of *P.gingivalis. T.denticola ,T.forsythia and A.actinomycetemcomitans.* Similar methods have been used by other laboratories with a good success. [**Ashimoto A et al 1996[2], Conrads G et al 1997[20].]**

Traditionally, subgingival plaque samples have been taken with either a curette or paper point and some variations have been documented between and within these sampling methods**.** [**Tanner AC et al 1986**[86]] Sampling by paper point is less invasive than by curette but may result in an underestimation of tightly adherent bacteria in subgingival sites. [**Hartroth B et al 1999**[40]] Hence in this study, curettes were used for sample collection.

In the present study, subgingival plaque samples and atheromatous plaque specimen were examined with the PCR technique using specific primers for periodontal pathogens. The DNA of atleast one of the target bacteria was detected in 96% of the subgingival sample. The prevalence of *P.gingivalis, T.denticola,T.forsythia* was 92% ,80%, and 96% respectively. The sequence of periodontal bacterial DNA in the atheromatous plaques could be ascertained by DNA sequencing and they were compared with type strains sequences from the Gen Bank by blast to confirm their sequence homology. the prevalence of *P.gingivalis, T.forsythia ,T.denticola* was 64%, 56% and 76% respectively. The DNA of periodontal bacteria except *A.actinomycetemcomitans* was detected in both subgingival samples and atheromatous samples. In contrast to the study by **Padilla C et al in 2006**[67] who showed the presence of *A.actinomycetem comitans* in atheromatous plaques and the periodontal pockets of the same patients and also stated that, this could indicate a role for periodontal pathogenic bacteria in the atherosclerosis disease process.

**Almetti M et al in 2007**[1] observed that, Bacterial DNA was detected in 31 out of 33 endarterectomy specimens. However, none of the samples tested positive for

DNA from periodontal pathogens. The presence of periodontal bacteria in atheromatous plaques was not confirmed by his investigation. But in contrast to this in this study in 16 of 25 cases, *P.gingivalis* was present in both subgingival samples and atheromatous plaque samples. *T.denticola and T.forsythia* were present in both subgingival samples and atheromatous samples in 19 and 14 cases respectively. The data of this study were consistent with those reported by **Haraszthy et al**[39] ( PCR – amplified 16S rDNA species –specific probes , 30% positive for T.forsythia,26% for *P.gingivalis*, 14% for *P. Intermedia*) **Ishiara et al**[46] (PCR –amplified 16S rRNA, 21.6% positive for *P.gingivalis* , 5.9% for *T.forsythia*) and **ZHANG Yung–ming et al**[94] ( PCR-amplified 16S r DNA , 31%positive for *T.forsythia*,12% for *F. nucleatum*,18% *P.intermedia* and 33% for *P.gingivalis*.

The most frequently identified bacteria were *P.gingivalis* and *T.denticola*. This data corroborates with the study by **Maciej Zaremba et al 2007**[58].

The presence of periodontal bacteria DNA in coronary atheromatous plaque and subgingival plaque samples of the same patients was confirmed by this study and thus a correlation was established between putative bacteria contributing to atheromatous plaque and species associated with periodontal diseases.

As a result of small number of patients included in this work, which is a major limitation of this study, it is not possible to obtain conclusive statistical results; nevertheless, the presence of *P.gingivalis, T.denticola* and *T.forsythia* in the periodontal samples and in the atheromatous plaques of the same patients, support the potential role of this periodontopathogenic bacterial species in some step of the

atherogenesis process or as a contributor of a different mechanism that worsens this disease. However, if this association is casual, a deeper evaluation of periodontal diseases is necessary. This work opens new insights regarding the potential risk of *P.gingivalis, T.denticola* and *T.forsythia* for atherosclerosis. Further molecular studies are required for a better understanding of this association.

# SUMMARY AND CONCLUSION

The study was performed on twenty five individuals with atherosclerosis. Atheromatous Plaques were collected from the patients during endarterectomy and the subgingival plaque was collected from the same patients with clinical attachment level ≥ 4mm. Total DNA isolation was done, the presence of 16S rDNA for *P.gingivalis,T.denticola, T.forsythia and A.actinomycetemcomitans* was determined using PCR technique. The products were sequenced and they were compared with type strains from Gen Bank by blast to confirm their sequence homology. McNemar's Test was used for statistical analysis.

The DNA of atleast one of the target bacteria was detected in 96% of thesubgingival plaque samples. The prevalence of *P.gingivalis, T.denticola, and T.forsythia* was 92%, 80% and 96% respectively. The prevalence of *P.gingivalis, T.forsythia, and T.denticola* in atheromatous plaque samples was 64%, 56% and 76% respectively. The DNA of periodontal bacteria except *A.actinomycetemcomitans* was detected in both subgingival samples and atheromatous samples. In 16 of 25 cases, *P.gingivalis* was present in both subgingival samples and atheromatous plaque samples at P value of 0.008. In 19 cases, *T.denticola* was present in both subgingival samples and atheromatous plaque samples at P value of 0.031 and *T.forsythia* were present in both subgingival and atheromatous plaque samples in 14 cases at P value of 0.016.

The presence of periodontal bacteria DNA in coronary atheromatous plaque and subgingival plaque samples of the same patients was confirmed by this study and thus a correlation was established between putative bacteria contributing to atheromatous plaque and species associated with periodontal diseases. The link found in this study supports the concept that there may be a true link between atherogenesis and periodontitis.

It is well known that bactermia can occur after procedures such as extraction, root canal treatment, and scaling. However, research results suggest that anaerobic bacteremia may also occur as a result of toothbrushing and probing of periodontal pockets. **(Heimdahl A et al 1990[41], Roberts GJ 1999[72], Roberts GJ et al 1992[73])** Nevertheless, this bacteremia does not pose any threat to a healthy host because, according to research by **Pallash and Slots 1996[68],** it is eliminated by the host's immune system within a few minutes. Our results support the possibility that bacteria associated with periodontitis can permeate into coronary vessels. Therefore, it seems important to limit this process. To achieve this goal, systematic dental-plaque elimination is crucial, as well as the elimination of retention points favouring its accumulation. The reduction of bacteria present in supra- and subgingival plaque may be an important prophylaxis for periodontal and coronary diseases.

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## PROFORMA

## MICROBIOLOGICAL ANALYSIS OF PERIODONTAL POCKET AND CORANARY ATHEROMATOUS PLAQUE IN ATHEROSCLEROTIC PATIENTS

NAME OP.NO DATE

AGE / SEX ADDRESS

CHIEF COMPLAINTS (CVS)

History of presenting illness :

Past Medical History :

If under Medication :

Duration of Medication :

H/O Professional cleaning : Yes / No

H/O Diabetes : Yes / No

H/O Bleeding Disorder : Yes /No

H/O Smoking : Yes /No

**General Examination**

BP :

Pulse :

Respiratory rate :

Weight :

Height :

BSA :

Past Dental History :

Local Examination :

No. of Teeth Present :

Extracted Teeth :

(Reason for Extraction :

When Extracted)

Decayed / Filled Teeth :

**Investigations**

**Blood** :

Total Serum Cholesterol :

Total triglycerides :

High density lipoprotein :

Low density lipoprotein is calculated using the formula

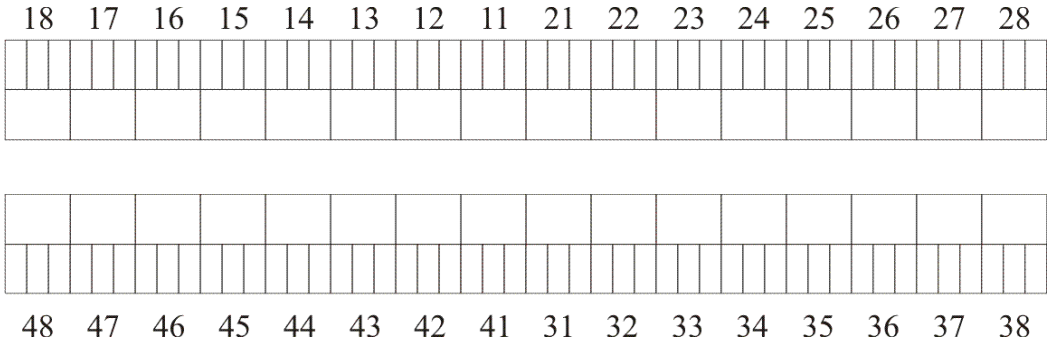
Low Density Lipoprotein = Total Serum Cholesterol – (Triglycerides / 2.2 + High Density Lipoprotein) in mgs/dl

- SGOT
- SGPT
- Alkaline Phosphatase
- CPK
- CPK – MB
- Trop
- Fasting blood glucose level
- Serum creatinine
- Haeomoglobin

- P.C.V.
- Platelete Count
- RBC Count
- WBC Count
- Neutrophils
- Lymphocytes
- Eosinophils
- E.S.R.
- B.T.
- C.T.
- P.T.
- INR
- A.P.T.T

**CLINICAL PARAMETERS:**

PLAQUE INDEX: (Silness & Loe 1964)




Plaque Index Score:_____

Inference: (Excellent/ Good / Fair / Poor)

**PROBING POCKET DEPTH [PPD], CLINICAL ATTACHMENT LEVEL [CAL]**

**(In mm):**

**Maxillary**

**BUCCAL**

| | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| CAL | | | | | | | | | | | | | | | | |
| PPD | | | | | | | | | | | | | | | | |
| | 18 | 17 | 16 | 15 | 14 | 13 | 12 | 11 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 |
| PPD | | | | | | | | | | | | | | | | |
| CAL | | | | | | | | | | | | | | | | |

**PALATAL**

**Mandibular**

**BUCCAL**

| CAL | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| PPD | | | | | | | | | | | | | | | | |
| | 48 | 47 | 46 | 45 | 44 | 43 | 42 | 41 | 31 | 32 | 33 | 34 | 35 | 36 | 37 | 38 |
| PPD | | | | | | | | | | | | | | | | |
| CAL | | | | | | | | | | | | | | | | |

**LINGUAL**

**PRESENCE OF 16S rDNA:**

| **Presence of 16S rDNA** | ***P.gingivalis*** | ***T.denticola*** | ***T.forsythia*** | ***A.actinomycete mcomitans*** |
|---|---|---|---|---|
| Subgingivalplaque | | | | |
| Atheromatous plaque | | | | |

## INFORMED CONSENT FORM-ENGLISH

Study Title:

**Microbiological analysis of periodontal pocket and coronary atheromatous plaque in atherosclerotic patients**

Date: O.P. No:

Name: Code No:

Address: Age/Sex:

Tel. No:

I, age yrs, exercising my free power of choice, hereby give my consent to be included as a participant in the clinical study "**Microbiological analysis of periodontal pocket and coronary atheromatous plaque in atherosclerotic patients**

I agree to the following:

I have been informed to my satisfaction about the purpose of the study, and nature of the study.

I understand that I should undergo endarterectomy procedure as a part of treatment for my condition in the heart.The atheromatous plaque sample taken during the procedure will be used for study purpose and serves the purpose of treatment and will not affect the treatment.

I understand that the investigations will also require oral plaque sample in required amounts.

I agree to co-operate fully and participate in the study.

I hereby give permission to use the records for study. I am told that study doctor and study institution will keep my identity confidential.

Signature of the Investigator

Signature of the Participant

# MuhŒ¢Á x¥òjš got«

**MuhŒ¢Á jiy¥ò**

**‘mÂnuh°»èbuho¡ nehahëfë‹ g‰gly« k‰W« fnuhdç mÂnuhkhl° gly« g‰¿a E©Qæçæaš MŒÎ’**

| | |
|---|---|
| ehŸ | òw nehahë v© |
| bga® | F¿p£bl© |
| Kftç | taJ / ghèd« |
| | bjhiyngÁ v© |

eh‹ ..... taJ ..... v‹Dila RaãidÎlD« KG RjªÂu¤JlD« ‘‘**mÂnuh°»èbuho¡ nehahëfë‹ g‰gly« k‰W« fnuhdç mÂnuhkhl° gly« g‰¿a E©Qæçæaš MŒÎ’** v‹D« ïªj kU¤Jt MuhŒ¢Áæš v‹id nr®¤J¡ bfhŸs r«kÂ¡»nw‹.

Ã‹ tUtd‰¿‰F eh‹ r«kÂ¡»nw‹:-

MuhŒ¢Áæ‹ neh¡f« k‰W« j‹ik F¿¤J vd¡F KG ÂU¥Âaë¡F«go és¡f¥g£lJ.

vdJ ÏUja¤Â‹ ãiyik F¿¤J, Á»¢iræ‹ xU gFÂahf v‹lh®blu¡lä brŒa¥gl nt©L« v‹gijÍ« vL¡f¥gL« mÂnuhkhl° gly« MŒÎ¡F¥ ga‹gL¤j¥gL« v‹gijÍ« ÏJ Á»¢iria v›éj¤ÂY« ghÂ¡fhJ v‹gijÍ« eh‹ cz®»nw‹.

gçnrhjid brŒtj‰fhf njitahd msÎ g‰gly« vL¡f¥gl nt©L« v‹gij eh‹ cz®»nw‹.

MŒÎ¡F v‹ KG x¤JiH¥igÍ« më¥ng‹ vd cWÂ TW»nw‹.

V‹Dil kU¤Jt¤ juÎfis MŒÎ¡F¥ ga‹gL¤Â¡ bfhŸs mDkÂ më¡»nw‹. vdJ Ra étu§fis kU¤JtU« MŒtfK« ÏufÁakhf it¤ÂU¥gh®fŸ vd m¿»nw‹.

| | | |
|---|---|---|
| MuhŒ¢Áahsç‹ ifbah¥g« | be¿ahsç‹ ifbah¥g« | g§F bgWgtç‹ ifbah¥g« |

# MASTER CHART

**TABLE - 1 General Characteristics and Presence of Micro Organisms in Sub Gingival Plaque**

| S.N | Age | Sex | Site | PPD | CAL | P.gingivalis | A.actinomycetemcomitans | T.denticola | T.forsythia |
|---|---|---|---|---|---|---|---|---|---|
| **1** | 58 | male | 16 mesial | 6 | 6 | + | - | - | + |
| **2** | 61 | male | 36 mesial | 5 | 5 | + | - | + | - |
| **3** | 54 | male | 16 mesial | 6 | 6 | + | - | + | + |
| **4** | 62 | female | 46 mesial | 7 | 7 | + | - | + | + |
| **5** | 51 | male | 36 mesial | 7 | 7 | + | - | + | + |
| **6** | 61 | male | 46 distal | 6 | 6 | + | - | + | + |
| **7** | 58 | female | 36 mesial | 7 | 7 | + | - | + | + |
| **8** | 68 | male | 36 distal | 7 | 7 | + | - | + | + |
| **9** | 43 | male | 46 distal | 6 | 7 | + | - | + | + |
| **10** | 59 | male | 36 distal | 5 | 5 | + | - | + | - |
| **11** | 57 | male | 46 distal | 7 | 7 | + | - | + | + |
| **12** | 41 | male | 46 distal | 7 | 7 | + | - | + | + |
| **13** | 60 | female | 36 distal | 7 | 7 | + | - | + | + |
| **14** | 53 | male | 46 distal | 6 | 6 | + | - | + | + |
| **15** | 63 | male | 45distal | 6 | 6 | + | - | + | + |
| **16** | 55 | female | 26 mesial | 6 | 6 | + | - | + | + |
| **17** | 66 | male | 36 distal | 7 | 7 | + | - | + | - |
| **18** | 60 | male | 17 mesial | 5 | 5 | - | - | + | + |
| **19** | 56 | male | 27 distal | 5 | 5 | + | - | + | + |
| **20** | 48 | male | 37 distal | 5 | 5 | + | - | + | - |
| **21** | 58 | male | 46 distal | 5 | 5 | + | - | + | + |
| **22** | 70 | female | 36 distal | 6 | 6 | + | - | + | + |
| **23** | 61 | male | 36 distal | 6 | 6 | + | - | + | + |
| **24** | 60 | male | 46 mesial | 5 | 5 | + | - | + | + |
| **25** | 47 | male | 36 mesial | 4 | 4 | - | - | + | - |

Present (+) Absent (-)

# MASTER CHART

**TABLE - 2 General Characteristics and Presence of Micro Organisms in Atheromatous Plaque**

| S.NO | Age | Sex | Site | CPK (U/L) | CPK-MB (U/L) | LDH (U/L) | P.gingivalis | A.actinomycetemcomitans | T.denticola | T.forsythia |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 58 | male | LIMA | 396 | 140 | 410 | - | - | - | - |
| 2 | 61 | male | LIMA-LAD | 234 | 306 | 22.7 | - | - | + | - |
| 3 | 54 | male | LIMA-LAD | 604 | 41 | 705 | - | - | - | - |
| 4 | 62 | female | LIMA-LAD | 485 | 43 | 638 | + | - | + | + |
| 5 | 51 | male | LIMA-LAD | 305 | 42 | 509 | + | - | + | + |
| 6 | 61 | male | LAD | 419 | 36 | 430 | - | - | + | + |
| 7 | 58 | female | LIMA-LAD | 816 | 98 | 682 | + | - | + | + |
| 8 | 68 | male | LIMA-LAD | 286 | 39 | 572 | + | - | + | + |
| 9 | 43 | male | LIMA-LAD | 284 | 34 | 514 | + | - | + | + |
| 10 | 59 | male | LIMA-LAD | 536 | 50 | 517 | - | - | - | - |
| 11 | 57 | male | LIMA-LAD | 306 | 39 | 503 | + | - | + | + |
| 12 | 41 | female | LIMA-LAD | 422 | 39 | 560 | + | - | + | - |
| 13 | 60 | male | RCA | 108 | 32 | 419 | + | - | + | + |
| 14 | 53 | male | LIMA-LAD | 393 | 43 | 812 | + | - | + | + |
| 15 | 63 | male | LIMA-LAD | 210 | 30 | 453 | - | - | - | - |
| 16 | 55 | female | RCA | 781 | 80 | 797 | + | - | + | - |
| 17 | 66 | male | LIMA | 468 | 40 | 615 | + | - | + | - |
| 18 | 60 | male | LIMA | 688 | 97 | 691 | - | - | + | + |
| 19 | 56 | male | LIMA | 456 | 84 | 687 | - | - | - | - |
| 20 | 48 | male | LIMA | 637 | 74 | 667 | + | - | + | - |
| 21 | 58 | male | LIMA-LAD | 498 | 66 | 517 | + | - | + | + |
| 22 | 70 | female | LIMA-LAD | 885 | 103 | 1020 | + | - | + | + |
| 23 | 61 | male | LIMA-LAD | 296 | 35 | 561 | + | - | + | + |
| 24 | 60 | male | RCA | 74 | 21 | 588 | + | - | + | + |
| 25 | 47 | male | LIMA-LAD | 310 | 39 | 575 | - | - | - | - |

Present (+) Absent (-)